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APPLICATION NUMBER: *60/435,817*
FILING DATE: *December 20, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/34683*

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

Compositions and Methods for Enzymatic Detachment of Bacterial Biofilms

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* **23**
☒ Drawing(s) *Number of Sheets* **2**
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☒ Other (specify) **Return postcard**

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

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Respectfully submitted,

SIGNATURE *Kathleen A. Tyrrell* Date **12/20/02**

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TELEPHONE **856-810-1515** Docket Number: **UMD-0010**

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

UMD-0010

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1) Patent Application Transmittal Letter (in duplicate);

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4) Two sheets of informal drawings containing Figures 1 and 2;

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Kathleen A. Tyrrell

UMD-0010 PATENT

Compositions and Methods for Enzymatic Detachment of Bacterial Biofilms

Field of the Invention

The present invention provides isolated nucleic acid sequences and amino acid sequences encoded thereby for a protein, soluble, β-N-acetylglucosaminidase or DspB, or an active fragment thereof which promotes detachment and/or release and dispersion of bacterial cells, particularly *Actinobacillus actinomycetemcomitans* cells and bacterial species related thereto, from biofilms. Vectors comprising the nucleic acid sequences as well as host cells expressing the DspB protein or active fragment thereof are also provided. In addition, a biofilm detachment mutant of *A. actinomycetemcomitans* is described. The nucleic acid and amino acid sequences of the present invention, as well as the biofilm detachment mutant of the present invention are useful in methods for modulating detachment and/or release and dispersion of bacterial cells from biofilms as well as in methods for identifying agents which modulate detachment and release and dispersion of bacterial cells from biofilms. Such agents are expected to be useful in the treatment of bacterial infections, particularly bacterial infection of the oral cavity.

Background of the Invention

Biofilms constitute a major portion of the bacterial biomass in most natural, industrial and clinical environments. Like all sessile organisms, biofilm bacteria must be able to release and disperse cells into the environment in order to colonize new sites. Proposed mechanisms for the detachment of cells from biofilms

include erosion and sloughing (Stodley et al. Appl. Environ. Microbiol. 2001 67:5608-5613). By erosion it is meant the continuous release of single cells or small clusters of cells and by sloughing it is meant the rapid detachment of large portions of the biofilm. Erosion and sloughing has been suggested to result from biofilm associated processes such as enzyme production (Allison et al. FEMS Microbio. Lett. 1998 167:179-184; Boyd et al. Appl. Environ. Microbiol. 1994 60:2355-2359; Lee et al. Infect. Immunol. 1996 64:1035-1038), chemical signal production (Puskas et al. J. Bacteriol. 1997 179:7530-7537), cell-cycle mediated events (Allison et al. J. Bacteriol. 1990 172:1667-1669; Gilbert et al. Appl. Environ. Microbiol. 1989 55:1308-1311; Gilbert et al. J. Appl. Bacteriol. 1993 74:67S-78S), and global regulation (Jackson et al. J. Bacteriol. 2002 184:290-301), or from external factors such as shear forces (Gilbert et al. J. Appl. Bacteriol. 1993 74:67S-78S; Picioreannu et al. Biotechnol. Bioeng. 2001 72:205-218), abrasion by collision of solid particles with the biofilm, and predator grazing (Stewart, P.S. Biotechnol. Bioeng. 1993 41:111-117).

Sloughing typically occurs in older biofilms (Peyton et al. Biotechnol. Bioeng. 1993 41:728-735) and is considered an important mechanism in the shedding of microcolonies from preformed biofilms on heart valves that often results in infective emboli that can lead to stroke (Donlan, R.M. and Costerton, W. Clin. Microbiol. Rev. 2002 15:167-193). Sloughing is also believed to be an important mechanism in the dissemination of *Legionella* from water-cooling systems (Lee, J.V. and West, A.A. J. Appl. Bacteriol. 1991 121S-130S).

Abrasion is believed to play a role in biofilm dispersal in the oral cavity.

Predator grazing is believed to play a role in environments such as streams and soil. Phagocytosis is a

form of predator grazing which can result in detachment and dispersal of cells from a biofilm. Detached cells can then be dispersed by passive means such as current flow or active means such as swimming motility or surface translocation (Pratt, L.S. and Kolter, R. Mol. Microbiol. 1998 30:285-293).

Several studies have also been conducted with various bacterial species which are indicative of cells being released from biofilms by the activity of endogenous enzymes. For example, overexpression of alginate lyase, an enzyme that catalyzes the degradation of the exopolysaccharide alginate, causes colonies of *Pseudomonas aeruginosa* to become less adherent to surfaces (Boyd, A. and Chakrabarty, A. M. Appl. Environ. Microbiol. 1994 60:2355-2359). Alginate is a polymer composed of mannuronic and guluronic acids that surrounds the bacteria and helps anchor them to the biofilm colony. Alginate lyase has been suggested for use in treating *P. aeruginosa* infections in the lungs of cystic fibrosis patients (Mrsny et al. Pulm. Pharmacol. 1994 7:357-366). A similar polysaccharide lyase has been shown to be produced by *P. fluorescens* (Allison et al. FEMS Microbiol. Lett. 1998 167:179-184). Cells of the gram-positive species *Streptococcus mutans* (Lee et al. Infect. Immun.1996 64:1035-1038; Vats, N. and Lee, S.F. Arch. Oral Biol. 2000 45:305-314) and the methanogenic archaebacterium *Methanosarcina mazei* (Liu et al. Appl. Environ. Microbiol. 1985 49:608-613) have also been shown to be released from biofilms by endogenous enzymatic activities. In the case of *M. mazei*, disaggregation is caused by an enzyme (disaggregatase) that degrades a heteropolysaccharide capsule that enmeshes the bacterial cells (Xun et al. Appl. Environ. Microbiol. 1990 56:3693-3698).

The present invention provides an isolated protein and nucleic acid sequence encoding this protein or a

fragment thereof involved in detachment of biofilm cells of *Actinobacillus actinomycetemcomitans*. Methods for modulating detachment of biofilm cells of bacteria and identifying agents which modulate detachment and/or dispersion and/or release via these protein and/or nucleic acid sequences are also provided.

Summary of the Invention

An object of the present invention is to provide an isolated protein which promotes detachment and/or release and dispersion of bacterial cells from a biofilm. The isolated protein is referred to herein as soluble, β-N-acetylglucosaminidase.

Another object of the present invention is to provide isolated nucleic acid sequences encoding soluble, β-N-acetylglucosaminidase and active fragments thereof as well as vectors comprising these sequences and host cells expressing the vectors.

Another object of the present invention is to provide methods for modulating detachment and/or release and dispersion of bacterial cells from biofilms. In one embodiment the method comprises mutating the bacterial cells to inhibit detachment and/or release and dispersion of bacterial cells from biofilms. In another embodiment, the method comprises increasing expression and/or levels of soluble, β-N-acetylglucosaminidase in the bacterial cells so that detachment and/or release and dispersion is increased. In yet another embodiment, the method comprises decreasing expression and/or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase so that detachment and/or release and dispersion of bacterial cells is decreased.

Another object of the present invention is to provide an isolated mutant of *Actinobacillus actinomycetemcomitans* which forms biofilm colonies which tightly adhere to

surface but which are unable to release cells into the medium or spread over the surface.

Another object of the present invention is to provide a method for identifying agents which modulate detachment and/or release and dispersion of bacterial cells from biofilms which comprises assessing the ability of an agent to modulate activity and/or levels and/or expression of soluble, β-N-acetylglucosaminidase.

Another object of the present invention is to provide compositions and methods for using these compositions to prevent the dissemination of infectious bacteria via administration of an agent which inhibits soluble, β-N-acetylglucosaminidase expression and/or activity in the bacterial cells.

Brief Description of the Figures

Figure 1 provides the nucleic acid sequence for *dspB* of *A. actinomycetemcomitans* (SEQ ID NO:1) which encodes soluble, β-N-acetylglucosaminidase, also referred to herein as DspB.

Figure 2 provides a comparison of the predicted amino acid sequence of *A. actinomycetemcomitans* DspB (top; SEQ ID NO:2) with the sequence of lacto-N-biosidase from *Lactococcus lactis* (GenBank accession no. AAK05592; bottom; SEQ ID NO:3).

Detailed Description of the Invention

The small, gram-negative coccobacillus *Actinobacillus actinomycetemcomitans* is a common inhabitant of the human oral cavity (King, E.O. and Tatum, H.W. J. Infect. Dis. 1962 111:85-94). *A. actinomycetemcomitans* has been implicated as the causative agent of localized juvenile periodontitis, a severe and rapid form of periodontal disease that affects adolescents (Zambon, J.J. J. Clin.

Periodontol 1985 12:1-20). *A. actinomycetemcomitans* can also enter the submucosa and cause infective endocarditis and other non-oral infections (Kaplan et al. Rev. Infect. Dis. 1989 11:46-63).

When cultured in broth, fresh clinical isolates of *A. actinomycetemcomitans* form tenacious biofilms on surfaces such as glass, plastic and saliva-coated hydroxyapatite (Fine et al. Arch. Oral. Biol. 1999 44:1063-1076; Fine et al. Microbiol. 1999 145:1335-1347; Fine et al. Arch. Oral Biol.2001 46:1065-1078; Haase et al. Infect. Immun. 1999 67:2901-2908; Inouye et al. FEMS Microbiol. Lett. 1990 69:13-18; Kachlany et al. J. Bacteriol. 2000 182:6169-6176; Kachlany et al. Mol. Microbiol. 2001 40:542-554; Kagermeier, A. S., and London, J. Infect. Immun. 1985 47:654-658; Kaplan, J. B., and Fine, D.H. Appl. Environ. Microbiol. 2002 68:4943-4950; King, E. O. and Tatum, H.W. J. Infect. Dis. 1962 111:85-94; Rosan et al. Oral. Microbiol. Immunol. 1988 3:58-63). Nearly all of the cells grow attached to the surface while the broth remains clear and is often sterile (Fine et al. Arch. Oral. Biol. 1999 44:1063-1076). The dense biofilm that forms on the surface is resistant to removal by agents such as detergents, proteases, heat, sonication and vortex agitation (Fine et al. Arch. Oral. Biol. 1999 44:1063-1076), and can be removed only by mechanical scraping. *A. actinomycetemcomitans* biofilm colonies exhibit increased resistance to antimicrobial agents when compared to cells grown in planktonic form (Fine et al. J. Clin. Periodontol. 2001 28:697-700).

Tight adherence has been shown to play an important role in the ability of *A. actinomycetemcomitans* to colonize the mouths of rats (Fine et al. Arch. Oral Biol. 2001 46:1065-1078.), and is believed to have an equally important role in its ability to colonize humans. The tight adherence to surfaces is dependent on the presence of long,

bundled pili (fimbriae) that form on the surface of the cell (Inouye et al. FEMS Microbiol. Lett. 1990 69:13-18; Rosan et al. Oral. Microbiol. Immunol. 1988 3:58-63). Mutations in *flp-1*, which encodes the major pilin protein subunit, result in cells that fail to produce fimbriae or adhere to surfaces (Kachlany et al. Mol. Microbiol. 2001 40:542-554).

Biofilm colonies of *A. actinomycetemcomitans* have been shown to release cells into liquid medium which then attach to the surface of the culture vessel and form new colonies, enabling the biofilm to spread (Kaplan, J. B. and Fine D. H. Appl. Environ. Microbiol. 2002 68: 4943-4950.).

One aspect of the present invention relates to a mutant of *A. actinomycetemcomitans* that forms biofilm colonies which are tightly adherent to surfaces but which are unable to release cells into the medium or spread over the surface. The biofilm detachment mutant of *A. actinomycetemcomitans* is referred to herein as mutant JK1023. To produce the *A. actinomycetemcomitans* biofilm detachment mutant JK1023, the *A. actinomycetemcomitans* strain CU1000N was mutagenized with transposon IS*903*φ*kan*. The mutant strain (designated JK1023) was then isolated. This mutant strain displays a colony morphology on agar that is rougher than the wild-type *A. actinomycetemcomitans* rough-colony phenotype (Fine et al. Microbiol. 1999 145:1335-1347; Haase et al. Infect. Immun. 1999 67:2901-2908; Inouye et al. FEMS Microbiol. Lett. 1990 69:13-18). JK1023 colonies had a hard texture and were extremely difficult to remove from the agar surface. When cultured in broth, strain JK1023 produced biofilm colonies which were similar in size and shape to those of the wild-type strain, but which failed to produce satellite colonies on the surface of the culture vessel. Adherence of JK1023 cells to polystyrene was equal to that of wild-type

strain CU1000N as measured by a 96-well microtiter plate binding assay.

To demonstrate that biofilm colonies of mutant strain JK1023 of the present invention were deficient in biofilm cell detachment, biofilm colonies were grown for 24 hours on polystyrene rods suspended in broth in the wells of a 24-well microtiter plate. The amount of biofilm cell detachment was then quantified by staining the bacteria growing on the bottom of the well with crystal violet. Colonization at the bottom of the well results from cells that detach from the biofilm colonies growing on the polystyrene rod and fall to the bottom of the well. In this assay, biofilm colonies of strain JK1023 produced significantly less growth on the bottom of the well than the wild-type strain ($P < 0.01$, unpaired two-tailed *t* test). These data indicate that mutant strain JK1023 exhibited a wild-type surface attachment phenotype but a decreased biofilm cell detachment phenotype when compared to the wild-type strain CU1000N.

Mapping of the transposon insertion of this mutant strain revealed the insertion to be in a 1,143 bp open reading frame designated herein as *dspB*. The *dspB* sequence of strain CU1000 (serotype f) is 99.1% identical to that of *A. actinomycetemcomitans* strain HK1651 (serotype b). Six of the ten observed base changes result in silent codon substitutions. The *dspB* gene from strain CU1000 was predicted to encode a protein, referred to herein as DspB, having 381 amino acid residues with a molecular mass of 43.3 kDa. The 5' end of *dspB* contained a predicted signal peptide, suggesting that DspB may be a secreted protein.

Accordingly, another aspect of the present invention relates to nucleic acid sequences encoding DspB or active fragments thereof as well as amino acid sequences of DspB and active fragments thereof. Also encompassed by the present invention are vectors comprising these nucleic acid

sequences as well as host cells comprising the vectors which express DspB or an active fragment thereof.

By the term "nucleic acid sequence" as used herein it is meant to include, but is not limited to, unmodified RNA or DNA or modified RNA or DNA. Thus, by nucleic acid sequence it is meant to be inclusive of single- and double-stranded DNA, DNA that is a mixture of single-and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules containing DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. Further, the DNA or RNA sequences of the present invention may comprise a modified backbone and/or modified bases. A variety of modifications to DNA and RNA are known in the art for multiple useful purposes. The term "nucleic acid sequence" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acid sequences, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The DNA sequence of *dspB* from strain CU1000 will be deposited into GenBank under accession no. AYXXXXX. The nucleic acid sequence for this DNA is depicted in Figure 1 as SEQ ID NO:1. Accordingly, a preferred isolated nucleic acid sequence of the present invention comprises SEQ ID NO:1.

The deduced amino acid sequence of DspB is shown in Figure 2 as SEQ ID NO:2. There are similarities between the amino acid sequence of DspB and the consensus sequence of the family 20 glycosyl hydrolase. More specifically, amino acid residues 40 to 297 of the predicted DspB protein sequence are homologous to the catalytic domain of the family 20 glycosyl hydrolases (NCBI Conserved Domain Database accession number pfam00728). This family of

enzymes includes bacterial chitinases, chitobiases and lacto-N-biosidases (Sano et al. J. Biol. Chem. 1993 268:18560-18566; Tews et al. Gene 1996 170:63-67; Tsujibo et al. Biochim. Biophys. Acta 1998 1425:437-440.), and eukaryotic hexosaminidases (Graham et al. J. Biol. Chem. 1988 263:16823-16829). The protein most closely related to *A. actinomycetemcomitans* DspB is lacto-N-biosidase of *Lactococcus lactis* (GenBank accession no. AAK05592), which displays 28% identity over 281 amino acid residues not counting gaps and terminal extensions. See Figure 2, SEQ ID NO:3. Similarity between DspB and lacto-N-biosidases is high in the regions surrounding Arg47 and the acidic amino acid pair Asp202 and Glu203. See Figure 2. These residues have been shown to participate in substrate binding and catalysis in other family 20 glycosyl hydrolases (Mark et al. J. Biol. Chem. 2001 276:10330-10337; Mark et al. J. Biol. Chem. 1998 273:19618-19624; Prag et al. J. Mol. Biol.2000 300:611-617). The C-terminal half of DspB contained three Trp residues that were conserved in *L. lactis* lacto-N-biosidase (positions 236, 279, and 353). Multiple Trp residues are present in the C-terminal regions of the catalytic domains of all family 20 glycosyl hydrolases (Graham et al. J. Biol. Chem. 1988 263:16823-16829; Tews et al. Gene 1996 170:63-67). These Trp residues line the part of the substrate binding pocket that is complementary to the hydrophobic surfaces of the hexosamine sugar ring (Tews et al. Nature Struct. Biol. 1996 3:638-648).

Thus, in a preferred embodiment an isolated amino acid sequence of the present invention comprises SEQ ID NO:2 or an active fragment thereof. Preferred active fragments are those comprising a portion of the amino acid sequence of SEQ ID NO:2 with similarities to the consensus sequence of the family 20 glycosyl hydrolase.

The DspB protein engineered to contain an

octahistidine metal binding site at its C-terminus was expressed in *E. coli*. The protein was purified by Ni-affinity chromatography and the DspB portion was cleaved from the hybrid protein using thrombin. Analysis of the purified cleaved DspB protein by SDS-PAGE revealed the protein to migrate with an apparent molecular mass of 41 kDa. The N-terminal sequence of DspB was XCVKGNSIYPQK (SEQ ID NO:4) (where X is an unidentified residue). This matched codons 22 to 33 of CU1000 *dspB*, thus indicating that the dipeptide Met-Asn was cleaved from the N-terminus of the DspB fusion protein when expressed in *E. coli*. Analysis of purified, cleaved DspB protein by mass spectrophotometry resulted in a single major peak with an apparent molecular mass of 41.5 kDa, consistent with the predicted molecular mass of 41.4 kDa for the cleaved and processed DspB protein. The yield of DspB expressed in *E. coli* was 10 mg of protein per liter of culture.

The ability of DspB to cleave the glycosidic linkages of various 4-nitrophenyl-labeled synthetic hexosamine substrates was tested in an *in vitro* enzyme assay. DspB showed specificity for the 1→4 glycosidic bond of β-substituted *N*-acetylglucosaminide, consistent with the known functions of other family 20 glycosyl hydrolases (Tews et al. Nature Struct. Biol. 1996 3:638-648). DspB showed no activity against α-substituted *N*-acetylglucosaminide, or against α- or β-substituted *N*-acetylgalactosamine.

The effects of DspB protein on biofilm cell detachment of *A. actinomycetemcomitans* mutant strain JK1023 were then examined. In these experiments, DspB protein was added to growth medium of mutant strain JK1023 to determine if addition of this protein restored release of cells into the medium and dispersion. Polystyrene rods containing biofilm colonies of strain JK1023 were suspended in broth

containing various amount of DspB, and the amount of biofilm cell detachment was measured by staining the bacteria growing on the bottom of the well with crystal violet as in Example 5. Purified DspB restored the ability of mutant strain JK1023 to release cells into the medium and colonize the bottom of the microtiter plate well in a dose dependent manner. Heat-inactivated DspB had no effect on biofilm cell detachment of strain JK1023.

The effects of DspB protein on detachment of preformed biofilm colonies of *A. actinomycetemcomitans* and other bacteria were also examined. In these experiments, addition of DspB caused the detachment of preformed biofilm colonies of wild-type strain CU1000. DspB (50 μg/ml) caused a 50% reduction in the amount of surface-associated bacteria after 6 hours. Further, analysis by light micrography showed that the surface of treated colonies became grainy and flocculent when compared to the smooth-textured biofilm colonies observed with mock-treated cells. Also, the surface of the culture vessel became covered with a similar grainy material which had a fibrous appearance under higher power. These findings are consistent with the observed reduction in adherence of preformed biofilm colonies treated with DspB.

DspB caused a similar reduction in biofilm density when tested against biofilm colonies of four phylogenetically diverse strains of *A. actinomycetemcomitans* representing four different serotypes, and a strain of the closely related bacterium *Haemophilus aphrophilus*. DspB did not cause the detachment of biofilm colonies of *Neisseria subflava*, *Cardiobacterium hominis* or *Streptococcus mitis*.

Thus, as demonstrated by these experiments, mutation of the dspB gene as well as addition of the isolated DspB protein modulates the detachment of cells from biofilm colonies.

Accordingly, the present invention also relates to methods for modulating detachment and/or release and dispersion of bacterial cells from biofilms.

In one embodiment, the method comprises mutating *dspB* of bacterial cells to inhibit detachment and/or release and dispersion of bacterial cells from biofilms such as in the JK1023 mutant of the present invention. In another embodiment, the method comprises decreasing expression and/or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in bacterial cells so that detachment and/or release and dispersion of bacterial cells is decreased.

The present invention also provides methods for promoting detachment and/or release and dispersion of bacterial cells from a biofilm which comprises contacting bacterial cells with soluble, β-N-acetylglucosaminidase or an active fragment or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment thereof. For example, *A. actinomycetomecomitans* Dsp was found to detach biofilms of *Haemophilus aphrophilus*. It is believed that biofilm detachment of *Actinobacillus pleuropneumoniae*, an important swine pathogen with a *dspB* homologue, will also be promoted in the presence of soluble β-N-acetylglucosaminidase or an active fragment thereof of the present invention.

The nucleic acid and amino acid sequences of the present invention, as well as the mutant JK1023 strain can also be used to identify agents which modulate detachment and/or release and dispersion of bacterial cells from biofilms. For example, the ability of an agent to modulate activity and/or expression of soluble, β-N-acetylglucosaminidase of the present invention can be assessed.

Examples of such agents include, but are not limited to antisense oligonucleotides or ribozymes targeted to the

dspB gene, peptidomimetics of DspB, and small organic chemicals which modulate DspB activity and/or levels and/or expression.

Agents which inhibit the ability of soluble, β-N-acetylglucosaminidase to promote detachment and release and dispersion of bacterial cells from biofilms are expected to be useful in preventing the dissemination of infectious bacteria, particularly infectious bacteria of the oral cavity such as *A. actinomycetemcomitans* and closely related bacterium such as *Haemophilus aphrophilus*.

The following nonlimiting examples are provided to further illustrate the present invention.

Examples

Example 1: Bacterial strains and growth conditions

A. actinomycetemcomitans CU1000 (serotype f) is a clinical strain isolated from a 13 year old patient with localized juvenile periodontitis (Fine et al. Microbiol. 1999 145:1335-1347). Strain CU1000N is a spontaneous nalidixic acid derivative of strain CU1000 that displays the same surface attachment, biofilm colony formation and biofilm dispersal phenotypes as the parental strain (Fine et al. Arch. Oral Biol. 2001 46:1065-1078; Kachlany et al. J. Bacteriol. 2000 182:6169-6176; Kachlany et al. Mol. Microbiol. 2001 40:542-554; Thomson et al. J. Bacteriol. 1999 181:7298-7307). Mutagenesis of strain CU1000N with transposon IS*903*φ*kan* was carried in accordance with the procedures set forth by Thomson et al. (J. Bacteriol. 1999 181:7298-7307). Other strains utilized include *A. actinomycetemcomitans* DF2200 (serotype a), NJ8800 (serotype b), NJ2700 (serotype c), and NJ9500 (serotype e) (Kaplan et al. J. Clin. Microbiol. 2002 40:1181-1187); *Haemophilus aphrophilus* NJ8700 (Kaplan et al. J. Clin. Microbiol. 2002 40:1181-1187); *Neisseria subflava* NJ9702 (Kaplan, J. B. and Fine, D.H.. Appl. Environ. Microbiol. 2002 68:4943-4950);

Cardiobacterium hominis NJ6500; and *Streptococcus mitis* NJ9705 (Kaplan, J. B. and Fine, D.H.. Appl. Environ. Microbiol. 2002 68:4943-4950). Bacteria were grown in Trypticase soy broth (BD Biosystems) supplemented with 6 grams of yeast extract and 8 grams of glucose/liter at 37°C in 10% CO_2.

Example 2: Cloning and sequencing *dspB*

The transposon insertion site in *A. actinomycetemcomitans* mutant strain JK1023 was cloned and sequenced by using an inverse PCR method in accordance with Kaplan et al. (Infect. Immun. 2001 69:5375-5384). The DNA sequence of the inverse PCR product was compared to the genome sequence of *A. actinomycetemcomitans* strain HK1651 from the Actinobacillus Genome Sequencing Project and the transposon was found to have inserted into a long open reading frame (ORF) which was designated *dspB*. This ORF corresponds to orf2128 in the Munich Information Center for Protein Sequences annotation of the HK1651 genome sequence. Primers that hybridize to sequences upstream and downstream from HK1651 *dspB* were used to amplify by PCR the *dspB* coding region from *A. actinomycetemcomitans* strain CU1000 using methods in accordance with Kaplan et al. (Infect. Immun. 2001 69:5375-5384). The forward primer (5-GCGCGCCATatgAATTGTTGCGTAAAAGGCAATTCC-3 (SEQ ID NO:5)) introduced an *Nde*I restriction site (underlined) and an ATG initiation codon (lower case) at codon positions 19 to 20 of *dspB*, and the reverse primer (5-GCGGTACCCTCATCCCCATTCGTCTTATGAATC-3 (SEQ ID NO:6)) replaced the *dspB* stop codon with a *Kpn*I restriction site (underlined). The PCR product (1,106 bp) was digested with *Nde*I and *Kpn*I and ligated into the *Nde*I/*Kpn*I sites of plasmid pET29b (Novagen). The insert of the resulting plasmid (designated pRC1) was subjected to DNA sequence analysis in accordance with procedures described by Kaplan

et al. (Infect. Immun. 2001 69:5375-5384).

Example 3: Expression and purification of recombinant DspB protein

Plasmid pRC1 carries a gene that encoded amino acids 21 to 381 of *dspB* fused to a 32 amino acid residue C-terminal tail containing an hexahistidine metal-binding site and a thrombin protease cleavage site which could be used to cleave the C-terminal tail from the hybrid protein. This gene was located downstream from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter.

Expression of DspB in E. coli

A one liter Erlenmeyer flask containing 500 ml of LB broth supplemented with 50 μg/ml of kanamycin was inoculated with 5 ml of an overnight culture of *E. coli* strain BL21(DE3) (Dubendorff, J. W. and Studier, F. W. J. Mol. Biol. 1991 219:61-68) transformed with pRC1. The flask was incubated at 37°C with agitation (200 rpm) until the optical density of the culture (measured at 280 nm) reached 0.6 (approximately 3 hours). IPTG was added to a final concentration of 0.2 mM and the flask was incubated for an additional 5 hours with agitation. The cells were harvested by centrifugation for 15 minutes at 6,000 x g and the cell pellet was stored at -80°C.

Protein purification

The cell pellet was thawed on ice and resuspended in 20 ml of lysis buffer [20 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate] containing 10 mg/ml lysozyme. The cell suspension was sonicated for 30 seconds at 50% capacity, 70% duty cycle in a Branson model 4550 sonicator equipped with a microprobe and then cooled on ice for 30 seconds. The sonication and cooling steps were repeated four additional times. The cells were pelleted by centrifugation as above and the supernatant was transferred to a new tube. The cell pellet was resuspended in 20 ml of lysis buffer

without lysozyme and five additional cycles of sonication and cooling were performed. The cells were pelleted by centrifugation and the supernatant was removed and transferred to a new tube. The two supernatants were combined and loaded onto a 3 ml bed volume Ni-affinity column (catalog no. 154-0990, Pharmacia) according to the instructions supplied by the manufacturer. The column was washed with 50 ml of wash buffer [50 mM MOPS (pH 8.5), 20 mM KCl] containing 5 mM imidazole, followed by 25 ml of wash buffer containing 50 mM imidazole and 25 ml of wash buffer containing 100 mM imidazole. Fractions (1.5 ml each) were collected during the final wash and assayed for the presence of the hybrid protein by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining in accordance with procedures described by Sambrook et al. (1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Fractions containing the protein were pooled and dialyzed overnight against water using a 10,000 MW cut-off dialysis membrane. The purified protein was digested with 5 units of thrombin (Novagen) per mg of protein for 1 hour at room temperature and the thrombin was removed using a Thrombin Cleavage Capture Kit (Novagen) according to instructions supplied with the kit. Undigested protein was removed by loading the sample onto a Ni-affinity column as described above and washing the column with 10 ml of wash buffer containing 5 mM imidazole. Fractions of the wash (1.5 ml each) were collected and analyzed for the presence of the protein by SDS-PAGE. Fractions containing the protein were pooled, dialyzed against water, and stored at -20°C.

N-terminal sequence analysis of the purified protein was carried out using the Edman degradation procedure on a Beckman model 2300 protein sequencer. Mass spectra were determined by using a Hitachi model 4414 mass spectrometer.

Example 4: Enzyme assays

Synthetic substrates (purchased from Sigma Chemical Co.) were 4-nitrophenyl-*N*-acetyl-β-D-galactosaminide, 4-nitrophenyl-*N*-acetyl-α-D-galactosaminide, 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide, and 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide. Enzyme reactions were carried out in a 10 ml volume containing 50 mM sodium phosphate buffer (pH 5.9), 100 mM NaCl, 5 mM substrate, and 3.7 μg/ml purified protein in a 15 ml polypropylene tube placed in a 37°C water bath. The reaction was terminated at various times by transferring 1 ml of the reaction mixture to a new tube containing 5 μl NaOH. The increase in absorption resulting from the release of *p*-nitrophenolate in each tube was measured in a Shimadzu UV-Mini spectrophotometer set to 405 nm.

Example 5: Detachment of biofilm cells from polystyrene rods

An assay to measure the detachment of cells from preformed biofilm colonies grown on polystyrene rods was carried out in 96-well microtiter plates. Biofilm colonies were grown on polystyrene rods suspended in broth in the 96-wells of a microtiter plate. Cells that detached from the biofilm fell to the bottom of the well where they attached to the surface and formed new biofilm colonies. The amount of biofilm growth on the bottom of the well, which was proportional to the number of cells that detached from the biofilm colonies on the rods, was measured by staining with crystal violet. The detachment assay was carried out as follows.

Construction of the apparatus

The lid of a 96-well polystyrene flat-bottomed tissue culture plate (Falcon No. 353072) was modified as follows: First, 96 1.5-mm diameter holes were drilled in the lid, with each hole in a position corresponding to the center of

one of the 96 wells. Then, an 11-mm long polystyrene rod (1.5-mm diameter, Plastruct Corp., City of Industry, CA) was placed in each hole (with one end of the rod flush against the top of the lid) and secured with trichloromethane plastic solvent. When this modified lid was placed on a 96-well microtiter plate bottom, the rods were suspended in the wells with the bottom of each rod approximately 2 mm above the bottom of the well. The modified lid was sterilized by soaking in 70% ethanol for 30 minutes and air drying in a biological safety cabinet.

Inoculation and incubation of polystyrene rods

The microtiter plate bottom was filled with medium (100 µl per well) and each well was inoculated with a single 2-3 day old colony from an agar plate using a sterile toothpick. The modified lid was then placed on the inoculated plate to submerge the polystyrene rods in the inoculated medium, and the plate was incubated at 37°C for 24 hours to allow that bacteria to adhere to the rods. The lid was then transferred to a fresh microtiter plate containing prewarmed medium and incubated for an additional 24 hours to allow biofilm cells to detach from the rods.

Measuring detached cells

The lid was removed and the plate was washed extensively under running tap water to remove loosely adherent cells. The wells were filled with 100 µl of Gram-staining reagent (2 grams crystal violet, 0.8 grams ammonium oxalate, 20 ml ethanol per 100 ml) and the plate was incubated at room temperature for 10 minutes. The plate was re-washed extensively under running tap water to remove unbound dye. The wells were than filled with 100 µl of ethanol and the plate was incubated at room temperature for 10 minutes to solubilize the dye. The optical density (at 590 nm) of the ethanol/dye solution in each well was measured using a Bio-Rad benchmark microplate reader.

Example 6: 96-well microtiter plate biofilm cell detachment assay

The wells of a 96-well microtiter plate (Falcon no. 353072) were filled with 100 μl of medium containing 10^2 to 10^4 CFU of bacteria and incubated at 37°C in 10% CO_2 for 20 hours. Ten μl of enzyme solution [1 mg ml^{-1} in phosphate buffered saline (PBS)], or 10 μl of PBS in the case of controls, was added to each well and the plates were incubated for an additional 6 hours. The wells were washed extensively under running tap water and the bacteria remaining attached to the surface were stained with crystal violet, rewashed, and destained with ethanol in accordance with procedures described by Kachlany et al.Mol. Microbiol. 2001 40:542-554). The optical density (O.D.) of the ethanol-dye solution was measured in a BioRad Benchmark microtiter plate reader set to 590 nm.

What is Claimed is:

1. An isolated nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment thereof which promotes detachment or release and dispersion of bacterial cells from a biofilm.

2. The isolated nucleic acid sequence of claim 1 comprising SEQ ID NO:1.

3. A vector comprising the nucleic acid sequence of claim 1.

4. A host cell comprising the vector of claim 3.

5. An isolated amino acid sequence encoded by the nucleic acid sequence of claim 1.

6. An isolated soluble, β-N-acetylglucosaminidase protein which promotes detachment and release and dispersion of bacterial cells from a biofilm.

7. The isolated soluble, β-N-acetylglucosaminidase protein of claim 6 comprising SEQ ID NO:2.

8. A method for inhibiting detachment or release and dispersion of bacterial cells from biofilm comprising mutating a *dspB* gene of bacterial cells to inhibit detachment and release and dispersion of bacterial cells from biofilms.

9. A method for inhibiting detachment or release and dispersion of bacterial cells from biofilm comprising decreasing expression or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in the bacterial cells so that

detachment or release and dispersion of bacterial cells from the biofilm is decreased.

10. An isolated mutant of *Actinobacillus actinomycetemcomitans* which forms biofilm colonies which tightly adhere to surface but which are unable to release cells into the medium or spread over the surface.

11. The mutant of claim 10 wherein the *dspB* gene is mutated.

12. A method for promoting detachment or release and dispersion of bacterial cells from a biofilm comprising contacting bacterial cells with soluble, β-N-acetylglucosaminidase or an active fragment thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment thereof so that detachment or release and dispersion of bacterial cells from a biofilm is promoted.

13. A method for identifying an agent which modulates detachment or release and dispersion of bacterial cells from biofilms comprising assessing an agent's ability to modulate activity or expression or levels of soluble, β-N-acetylglucosaminidase.

14. A composition for preventing dissemination of infectious bacteria comprising an agent which inhibits soluble, β-N-acetylglucosaminidase expression or activity or decreases levels of soluble, β-N-acetylglucosaminidase in the bacterial cells.

15. A method for preventing dissemination of infectious bacteria comprising administering to the bacteria the composition of claim 14.

ABSTRACT

Isolated nucleic acid sequences and amino acid sequences for soluble, β-N-acetylglucosaminidase or an active fragment thereof which promotes detachment and release and dispersion of bacterial cells from a biofilm are provided. An isolated mutant bacteria which forms biofilm colonies which tightly adhere to surface but which are unable to release cells into the medium or spread over the surface is also provided. In additions, methods are described for modulating detachment or release and dispersion of bacterial cells from biofilm by mutating soluble, β-N-acetylglucosaminidase or altering its expression or activity are also provided. Also provided are compositions and methods for using compositions for preventing dissemination of infectious bacteria containing an agent which inhibits soluble, β-N-acetylglucosaminidase expression or activity in the bacterial cells.

AATTGTTGCGTAAAAGGCAATTCCATATATCCGCAAAAAACAAGTACCAAGCAGACCGGATTAAT
GCTGGACATCGCCCGACATTTTTATTCACCCGAGGTGATTAAATCCTTTATTGATACCATCAGCC
TTTCCGGCGGTAATTTTCTGCACCTGCATTTTTCCGACCATGAAAACTATGCGATAGAAAGCCAT
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TTGAGTTGATTCCCGAACTTGACAGCCCGAATCACATGACGGCGATCTTTAAACTGGTGCAAAAA
GACAGAGGGGTCAAGTAGCTTCAAGGATTAAAATCACGCCAGGTAGATGATGAAATTGATATTAC
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CGAGTCAGCATTTTCATATTGGTGGCGATGAATTTGGTTATTCTGTGGAAAGTAATCATGAGTTT
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GAGTTGCTGGCGAAAGGCTTTACTGTCCTGAACTATAATTCCTATTATCTTTACATTGTTCCGAA
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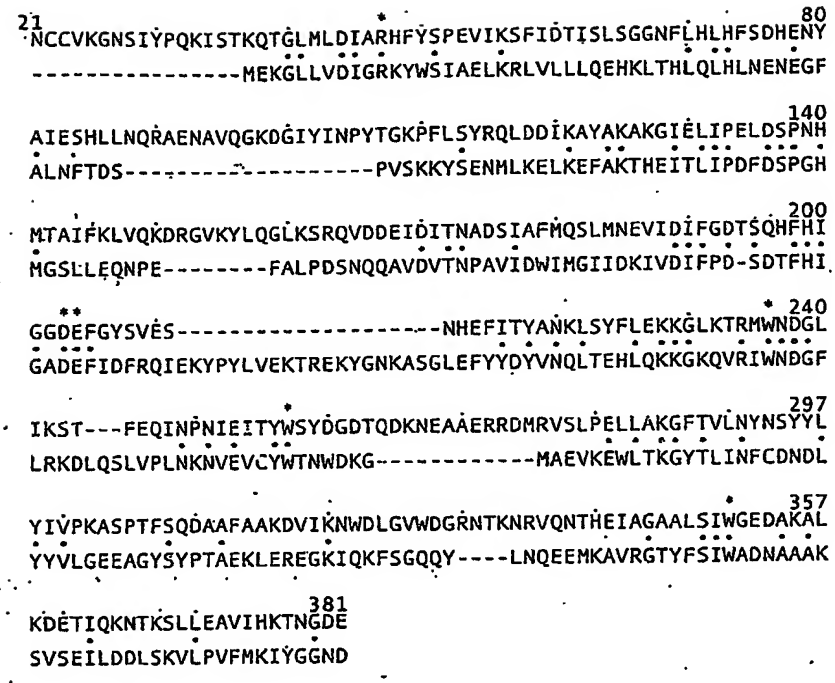
FIGURE 1


21
80
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140
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ALNFTDS----------PVSKKYSENMLKELKEFAKTHEITLIPDFDSPGH
200
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240
GGDEFGYSVES----------NHEFITYANKLSYFLEKKGLKTRMWNDGL
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297
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357
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381
KDETIQKNTKSLLEAVIHKTNGDE
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FIGURE 2